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Nadezda Lipunova[1,2,5], Anke Wesselius[2], Kar K. Cheng[3], Frederik-Jan van Schooten[4], Richard T. Bryan[1], Jean-Baptiste Cazier[1,5], Tessel E. Galesloot[6], Lambertus A. L. M. Kiemeney[6], Maurice P. Zeegers[1,2]

[1]Institute of Cancer and Genomic Sciences, University of Birmingham, United Kingdom, B15 2TT; Email: nxl689@student.bham.ac.uk, n.lipunova@maastrichtuniversity@nl; Tel. nr.: +4429189097

[2]Department of Complex Genetics, Maastricht University, The Netherlands

[3]Institute for Applied Health Research, University of Birmingham, United Kingdom

[4]Department of Pharmacology and Toxicology, Maastricht University, The Netherlands

[5]Centre for Computational Biology, University of Birmingham, United Kingdom

[6]Radboud University Medical Center, Radboud Institute for Health Sciences, The Netherlands

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ABSTRACT

Background

Non-muscle-invasive bladder cancer (NMIBC) causes a considerable health burden due to the high recurrence and progression rates. Past studies have identified multiple candidate loci associated with NMIBC prognosis, albeit lacking validation. Moreover, scarce reports exist on genetic susceptibility to independent prognostic predictors of NMIBC, such as stage or grade.

Objective

To investigate genetic associations with NMIBC tumour and patient characteristics at the time of diagnosis.

Design, Setting, and Participants

A sample of 653 NMIBC cases come from the Bladder Cancer Prognosis Programme (BCPP). Replication of the significant findings was conducted in the Nijmegen Bladder Cancer Study (NBCS) cohort (N=1470).

Outcome Measurements and Statistical Analysis

Genome-wide association study (GWAS) was carried out for outcomes of tumour size (as continuous variable in centimetres), stage (Tis and T1 vs Ta), grade (G3 vs G2 and G1), and age (as continuous (years) and dichotomous (70.2 years as a cut-off) variables).

Results and Limitations

Significant (P<5E-08) associations (N=61) with tumour size, stage, grade, and age were identified in the GWAS discovery stage. None of the variants were independently significantly associated in the replication cohort. A meta-analysis of both cohorts suggests rs180940944 (13q13.3 locus, *NBEA*) was associated with tumour size as a continuous

variable (ß=0.9 cm, p=2.92E-09). However, other SNPs in this region did not show evidence of association in the meta-analysis.

Conclusions

Our study suggests rs180940944 (*NBEA*) is associated with an increased NMIBC tumour size at the time of diagnosis. Given study limitations, further replication is essential to validate the finding.

Patient Summary

Current study reports on a genome-wide association study on non-muscle-invasive bladder cancer tumour and patient characteristics. We suggest *NBEA* gene might be associated with increased tumour size at the time of diagnosis. The result must be replicated to establish validity.

INTRODUCTION

Urinary bladder cancer (UBC) accounts for 430 000 new cases worldwide annually, with 70-80% of new cases presenting as non-muscle-invasive bladder cancer (NMIBC) [1]. NMIBC causes significant burden on healthcare systems due to high recurrence and progression rates (5-year recurrence rate: 50-70%, 5-year progression rate: 10-30%) [1]. Considerable clinical improvements could be made by better, even personalised, prognostication and risk stratification [1]. There have been several attempts to apply different approaches for accurate disease prognostication, and although descriptive on a population-level, a substantial lack of precision of individual outcomes remains [2], requiring ongoing improvement.

Few candidate-gene studies of UBC prognosis exist, with limited successful replication [3-5]. A recent study reported that out of 114 reported loci for UBC progression and prognosis, only six single nucleotide polymorphisms (SNPs) showed significant associations in an independent cohort, namely: NMIBC progression (rs6678136 (RGS4), rs11585883 (RGS5)), recurrence among Bacillus Calmette–Guérin (BCG)-treated NMIBC patients (rs1799793 (ERCC2), rs187238 (IL18)), and muscle-invasive bladder cancer (MIBC) overall survival (rs12035879 (RGS5), rs2075786 (TERT)) [3]. Powerful GWAS studies on NMIBC prognosis show promise, but are still ongoing [6].

A previous attempt to include genetic variation failed to increase prognostic tool performance [7], suggesting the issue is more complex. However, latter study utilised a relatively small panel of SNPs (170,000), which has lower power of discovering significant loci in comparison to genotype-imputed sets harbouring millions of variants for analysis [8]. The inter-study lack of consensus might be due to several reasons: spurious findings, lack of statistical power, and variation in outcome definition.

Other studies also suggest significant genetic signals might be only present for tumours of certain grade or stage [9, 10]. However, reports on genetic associations for characteristics that

directly influence NMIBC outcome are scarce, precluding further investigations on their relevance for NMIBC prognostication.

To provide more evidence on potential genetic associations, we have performed a GWAS on key NMIBC characteristics (stage, grade, size of the tumour, EORTC risk category), as well as age at the time of diagnosis within the West Midlands' Bladder Cancer Prognosis Programme (BCPP) cohort including replication in the Nijmegen Bladder Cancer Study (NBCS).

MATERIAL AND METHODS

Participants and genotyping

BCPP is a prospective cohort that initially recruited 1,544 eligible patients and is described in more detail elsewhere [11]. Clinical data on stage, grade, and size of tumours and demographic information (age, gender) were gathered with bespoke case report forms. Tumour size of the largest tumour was established visually while performing cystoscopy. Blood samples of 888 participants with confirmed UBC were genotyped on the Illumina Infinium OmniExpress-24 BeadChip array at deCODE Genetics (Iceland).

Tumours of stages pTa, pT1, or pTis were included to limit our analyses to NMIBC, resulting in a dataset of 712 cases.

Quality control (QC)

QC procedures were carried out using PLINK v1.90 [12]. The exact thresholds applied and number of exclusions per step are outlined in **Figure 1**.

Generic QC procedures per individual excluded those with an inconclusive gender call, excessive genotype missingness rate, increased or reduced genotype heterozygosity rate, duplicate samples, and related individuals.

To avoid any bias introduced by population stratification, a principal component analysis (PCA) was carried out. Investigation of PCA plots resulted in exclusion of clear population outliers. Genomic inflation factor (λ) value was estimated for all outcomes of interest; none of the values exceeded 1.03.

Marker-specific QC procedures covered excluding SNPs deviating from the Hardy-Weinberg equilibrium, exceeding acceptable missing rate, and rare variants.

In total, a dataset consisting of 653 individuals and 597,764 markers remained for further analyses.

Imputation

Imputation utilised a two-step approach: haplotype phasing by Eagle v2.3.2 [13], followed by genotype imputation with IMPUTE2 [14], using 1000 Genomes Phase 3 [15] as a reference panel in the genome build 19 (GRCh37/hg19). Once imputed, the dataset was filtered for SNPs with info values (an imputation accuracy measure) of >0.3 and MAFs of >1%, resulting in a dataset containing 11,914,228 markers available for genetic association analyses.

Statistical analysis

Statistical analyses were performed using SNPtest v2.5.2 [8] and R statistical package (v3.3.2) [16].

To establish the relation between germline variation and tested outcomes, linear regression was used for continuous variables and logistic regression for all binary endpoints. Age was tested as a continuous (years) and binary variable (mean was considered as a cut-off value for categorisation (resulting in strata of </≥ 70 years)). Tumour size (cm) was tested as a continuous and categorical variable (</≥ 3cm [17]). Stage (Tis and T1 versus Ta) and grade (G3 versus G2 and G1) were treated as binary variables. In addition, low-, intermediate-, and high-risk EORTC categories were assigned to each NMIBC case and were tested as a dichotomous variable of high- versus low- and intermediate-risk groups [17].

All analyses were adjusted for participant gender and first five genetic principal components to increase estimate precision and to adjust for any potential residual population stratification bias. An association was held significant if p-value <5E-08, and promising if below 5E-06.

Post-GWAS power calculations were carried out in web-based GAS Power Calculator [18].

Manhattan and QQ graphs were plotted for each tested outcome. For significant hits, regional association plots were constructed using LocusZOOM tool [19], except for hits that have not yet been assigned an ID (rsID).

Functional annotation

Identified significant SNPs were mapped using a web-based SNPnexus tool [20], with Ensembl [21] (Version 74) as a functional annotation system.

Replication

Genome-wide significant hits were attempted to replicate in a sample of 1470 NMIBC cases from the NBCS [22] (**Figure 1**). Briefly, the NBCS recruited UBC patients via the population-based cancer registry in the Nijmegen region. Eligible cases were diagnosed during 1995-2006 and were under the age of 75; additional data was collected via linkage with hospital-patient records [22], including tumour size, which was reported after visual evaluation during cystoscopy. Details of genotype data cleaning and initial analysis is provided in detail elsewhere [22].

We used META [23] software to perform meta-analysis on association results of both cohorts and calculated a combined p-value per SNP. An inverse-variance method was used, assuming a random-effects model. I^2 index and p-value were calculated to evaluate potential heterogeneity between the estimates of the two cohorts [23].

RESULTS

Baseline clinical characteristics of the discovery and replication cohorts are shown in **Table 1.**

Majority of cases in BCPP were male (78.1%), with an average age of 70 years. Tumour size mean was 2.5 cm, and most of the participants were diagnosed with stage Ta (68%) and T1 (30.5%) tumours. More than a third of cases presented as G2 (37.5%), followed by G3 (31.7%) and G1 (29.2%) NMIBC. The distribution of variable categories and measures were similar between the BCPP and NBCS cohorts.

In the discovery-stage analysis, a total of 61 SNPs, corresponding to 29 different regions, showed genome-wide statistically significant associations with at least one of the outcomes. Out of those, 20 loci were mapped to genes (all intronic regions) (**Table 2**). Significant associations were observed for size and age as continuous variables, as well as for binary outcomes of stage, grade, and age.

Most of the SNPs (N=47) were found to be associated with tumour size, the effect sizes ranging from 0.65 (rs35225990 in *FAM194B*, p= 2.85E-08) to 2.6 (rs370572716 in 9p13.1, p=4.04E-09) centimetres (**Table 2**).

One SNP in 9q22.32, rs142492877, showed statistically significant association with decreased age at diagnosis of almost one year (ß=-0.95, SE=0.16, p=1.05E-08). Age as a binary trait showed associations in the same direction, although in a different genomic region (7q31.33) with an odds ratio (OR) ranging between 2.46 (rs17149580, p=2.18E-08) and 2.51 (rs17149636, p=1.62E-08) across eight SNPs.

The 14q11.2 locus showed strong associations with being diagnosed with a higher grade of NMIBC (rs15091489 in the *TRAV16* gene (OR=3.42, 95%CI: 2.11-5.55, p=5.13E-09) and rs116923391 (OR=3.86, 95% CI: 2.38-6.26, p=2.07E-10)).

Several protective variants for tumour stage were observed, namely: rs117248430 in *ANKS6* (OR=0.003, 95%CI=1.71E-09-3895.6, p=3.73E-08), and two markers in the *SLCO1B1* gene (rs76497895 (OR=0.03, 95%CI=0.001-0.83, p=4.18E-08); rs116946525 (OR=0.03, 95%CI=0.001-0.83, p=4.23E-08)). The strength of the effect and corresponding confidence intervals in ANKS6 might be explained by a very low MAF (<0.01%) among cases.

A Manhattan plot for tumour size as a continuous outcome (**Figure 2**) also shows there are several polymorphisms in linkage disequilibrium (LD) with the leading SNP (Manhattan plots for all other tested outcomes are available in the **Supplementary Figures 1-6**).

Regional association plot of 13q13.3 (**Figure 3**) in the BCPP confirms high LD with surrounding variants, all mapping to the *NBEA* gene (although they did not reach the statistical significance). Regional association plots for the remaining SNPs identified in the discovery stage are presented in **Supplementary Figures 7-33**.

In the replication stage, 50 out of 61 SNPs were available to test in NBCS (**Table 2**). None of these SNPs were significantly associated with the same outcomes in NBCS. A meta-analysis of both cohorts showed variant rs180940944 in 13q13.3 locus to be associated with increased tumour size at diagnosis (ß=0.96, SE=0.16, p=2.92E-09), although the effect is likely driven by BCPP data. Nevertheless, low I^2 estimate (I^2=0%, p(heterogeneity)=0.75) indicated there was no significant heterogeneity between the two cohorts for the replicated SNP. A conditional association analysis on rs180940944 showed the associations in the *NBEA* gene are likely to be driven by the top SNP, as none of the variants have reached genome-wide significance when controlled for the effect of rs180940944 (**Supplementary Figure 34).** Nevertheless, the analysis also suggests there is a region in the *NBEA* gene of mildly inflated p-values, independent of the rs180940944.

DISCUSSION

We have investigated genetic associations with NMIBC tumour (size, stage, grade) and patient (age, EORTC risk category) characteristics at the time of diagnosis within the BCPP cohort.

Multiple loci were identified in the discovery stage that are novel in the context of NMIBC. One SNP, rs180940944, has reached statistical significance in a meta-analysis of two NMIBC cohorts, mapping to the intronic region of the *NBEA* gene on 13q13.3. However, associations of other SNPs in the *NBEA* have failed to be reproduced.

NBEA proteins have been mostly observed to play a significant role in synapse development and function [24]. *NBEA* dysregulation does not affect the establishment of synapses *per se*, but rather their intra-cellular organisation [24]. An in-depth analysis revealed impaired synaptic ability was mostly due to the inappropriate distribution of actin, a protein essential for synapse cytoskeleton structure [24]. The effect is most likely present due to alterations in the Golgi-dependent processes of inter- and intra-cellular compound trafficking, including actin and neural receptors [24].

The synaptic alterations are likely to be the contributing cause of autism spectrum disorders [24]; however, the Golgi-related pathway may have a wider phenotypic manifestation [25], including cancer. The prognostic utility of *NBEA* has been investigated in gastric cancer [26] and oropharyngeal squamous cell carcinomas (OPSCC) [27], with promising results. Collectively, these observations implicate the pleiotropic nature of *NBEA* effect across a variety of traits.

In our study, we suggest there is an association between *NBEA* and increased NMIBC tumour size. The role of Golgi complex in cancer progression has been reported independently, and disruptions in normal protein transportation can contribute to increased tumour size and, eventually, progression [25].

Our findings should be interpreted cautiously. Substantial sample sizes of specific phenotypes such as ours are rare, and suffer from limited power to capture true genetic associations, and spurious associations due to random effects cannot be ruled out. Our post-hoc power calculations [18], underscore the importance of current analysis being ran on bigger cohorts (e.g. association rs150914897 (14q11.2) of an OR=3.42 had power of 79%, but it drops to only 16% for an OR=2.5, hence we may have missed existing associations of more modest effect size).

Furthermore, tumour size measurements are subject to variability, degree of which is difficult to establish. The lack of any genome-wide significant associations for categorised tumour size (</≥ 3 cm [17]) adds substantial caution in consideration of our main findings and study power. However, clinically-relevant tumour size categories may not be adequate in a genetic context, and different categorisation may be used in future analyses.

Our study only focused on NMIBC instead of a merged group of UBC, and we are unable to comment on whether these genetic loci are relevant for advanced UBC. Given considered limitations, we see this study as true to the GWAS design of hypothesis-generating nature, instead of one offering conclusive findings. Hence, further replication is of essence to establish validity of described results.

The 13q13.3 locus has not been observed in prior studies on NMIBC. It might be due to us using an independent prognostic marker of NMIBC (i.e. tumour size) instead of recurrence and/or progression as an outcome. Larger tumour indicates a worse disease course [17], but there are other components that contribute to NMIBC prognosis. In a clinical setting, each tumour characteristic (e.g. size) carries a different weighting [17], collectively contributing to an endpoint (e.g. recurrence).

Importantly, powerful studies on UBC risk have already shown some signals to only be associated with MIBC (UBC of T2-T4) [10]. Furthermore, a genome-wide methylation

investigation on high-grade NMIBC cases revealed epigenetic changes different from their low-grade counterparts [9]. Direct comparability of these reports is limited, but we see the unravelling genetic complexity within UBC being a connecting thread between all studies. We therefore believe it is likely separate genetic relationships are present for NMIBC determinants, rather than overall prognostic outcomes.

CONCLUSIONS

Our study suggests variations in 13q13.3 locus may contribute to an increased NMIBC tumour size in a European population. Further studies are warranted to confirm the association.

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